Effect of Cardiolipin Oxidation on Solid-Phase Immunoassay for Antiphospholipid Antibodies

Michael Schlame, Ivan Haller, Lisa R. Sammaritano, Thomas J. J. Blanck

Department of Anesthesiology and the Division of Rheumatic Diseases, Hospital for Special Surgery, and the Biopolymer Mass Spectrometry Core Facility, Weill Medical College of Cornell University, New York, USA

Keywords

Anticardiolipin antibodies, antigen specificity, antiphospholipid syndrome, enzyme-linked immunosorbent assay, lipid oxidation

Summary

Diagnostic assays for antiphospholipid antibodies are routinely performed on microtitre plates coated with cardiolipin. Here we show that contact between cardiolipin and NUNC-Immuno® plates leads to extensive oxidation, generating a series of peroxy-cardiolipins which were identified by electrospray ionization mass spectrometry. To investigate the impact of oxidation on the antibody assay, cardiolipin was resolved into 12 molecular species, including oxidized species and non-oxidized species with different degrees of unsaturation. All 12 species reacted under anaerobic conditions with serum from patients with primary antiphospholipid syndrome. Immune reactivity was similar for reacted under anaerobic conditions with serum from patients with primary antiphospholipid syndrome. Immune reactivity was similar for

Introduction

The antiphospholipid syndrome (APS) is characterized by episodes of arterial, venous, or small vessel thrombosis and/or the presence of recurrent abortion (1, 2). Affected patients have either immunoglobulin G (IgG) or immunoglobulin M (IgM) anticardiolipin or the so-called “lupus anticoagulant” (3). It has been recognized that these antibodies play an active role in the pathogenesis of the disease, and thus distinguish it from other conditions associated with thrombosis or fetal loss (4).

Screening for antiphospholipid antibodies is generally done by solid-phase immunoassay using cardiolipin-coated microtitre plates. This assay has been widely criticized for lack of specificity and for obscuring the pertinent antigen responsible for the autoimmune disease. Conflicting evidence has suggested that antiphospholipid antibodies are directed either against cardiolipin and related anionic phospholipids, or against β2-glycoprotein I and other phospholipid-binding plasma proteins. While there may be true heterogeneity between antibody populations, even within a single patient, β2-glycoprotein I is now widely recognized as the target antigen of most antiphospholipid antibodies (5). Antibodies bind to the first domain of β2-glycoprotein I (6), even in the absence of phospholipids (7), albeit phospholipids may enhance the immune reaction by immobilizing β2-glycoprotein I on a solid support (8).

Recently it was proposed that the pertinent antigen of antiphospholipid antibodies is in fact generated when serum proteins, such as β2-glycoprotein I, are exposed to oxidized phospholipids (9, 10). This idea provided a working hypothesis to explain why autoantibodies exist against ubiquitous compounds such as phospholipids and serum proteins. It holds that these molecules only become antigens if they are chemically modified by oxidation. The oxidation hypothesis may explain why antiphospholipid titres rise in conditions associated with oxidative stress. For instance, cigarette smoking promoted the formation of anticardiolipin antibodies, and the increased titres were inversely related to the plasma level of the antioxidant vitamin C (11). In lupus erythematosus, antiphospholipid-positive patients showed increased urinary excretion of isoprostanes, a class of oxidized lipids derived from arachidonic acid (12). Acute infection is another example where increased oxidative stress is associated with the frequent occurrence of antiphospholipid antibodies (13).

The oxidation hypothesis has practical implications for the antibody assay because lipids may oxidize to various degrees on microtitre plates. If degree and kind of oxidative modification directly affect the assay, large inter-laboratory variations may occur with respect to antibody titre and binding specificity. In fact, it has been notoriously difficult to standardize the solid-phase assay for anticardiolipin antibodies and that in turn has hampered patient management and data communication for clinical studies (4). Therefore it is desirable to characterize chemical changes that occur in cardiolipin during solid-phase assay and to define the specificity of the immunoassay for molecular species with various modifications in the cardiolipin acyl groups.

Patients, Materials and Methods

Patients. Serum was obtained from 8 female patients diagnosed with primary APS. Diagnosis of APS was established in accordance with the preliminary classification criteria established by an international symposium on antiphospholipid antibodies (3). These criteria include at least one clinical symptom (vascular thrombosis or pregnancy morbidity) combined with the presence of moderate to high titres of anticardiolipin antibodies or lupus anticoagulant in plasma. Mean age was 38 years and the range was between 24 and 50 years. Serum was obtained for the purpose of clinical management and aliquots were stored frozen in an institutional collection of serum samples. Patients consented to the use of serum aliquots for research through participation in the Autoimmune Registry.
Immunoassay for human IgG antibodies. Ninety-six-well microtitre plates (Nalge Nunc International, Denmark) were coated with cardiolipin (2 μg/well). Cardiolipin was applied in ethanol that was quickly evaporated under a stream of nitrogen. Immediately after cardiolipin had dried, the plates were washed three times with phosphate-buffered saline (0.15 M per well), containing 8 g/L NaCl, 0.2 g/L KCl, 0.2 g/L KH₂PO₄, 0.2 g/L Na₂HPO₄, and 1.15 g/L Na₂HPO₄. Blocking was performed with 10% calf bovine serum in phosphate-buffered saline (0.125 M per well) for 1 h at 4°C. After blocking plates were washed twice with phosphate-buffered saline (0.15 M per well) and 0.1 M patient serum (diluted 1:100 with 10% calf bovine serum in phosphate-buffered saline) were added. Plates were incubated with serum for 2 h and 15 min at 4°C. After incubation plates were washed three times with phosphate-buffered saline (0.15 M per well) and 0.1 M of secondary antibody solution (alkaline phosphatase-labeled anti-human-IgG antibody, Sigma product A-3150, diluted 1:1000 with 10% calf bovine serum in phosphate-buffered saline) were added per well. Plates were incubated for 1 h at room temperature and subsequently washed three times with phosphate-buffered saline (0.15 M per well). Substrate solution for alkaline phosphatase (1 tablet of Sigma 104 Phosphate Substrate per 5 mL of 1 mol/L diethanolamine, pH 9.8) was added (0.1 M per well) and the plates were incubated in the dark for about 15 min until the yellow color developed. The reaction was analyzed with a Bio Rad 3550 microplate reader, measuring absorbance at 405 nm and 655 nm, respectively. The difference A₄05-A₆55 was proportional to the immobilized activity of alkaline phosphatase which in turn represented the amount of bound IgG.

Immunoassay for mouse IgM antibodies. Binding of the monoclonal antibody A1.72, derived from lupus-prone NZWxBXSB, F1 hybrid mice (14), was measured as described above with the following exceptions. Blocking was performed with 3% bovine serum albumin in phosphate-buffered saline. In all other steps, 1% bovine serum albumin was used instead of 10% calf bovine serum. Alkaline phosphatase labeled anti-mouse-IgM (Sigma product A-7784) was used as secondary antibody.

Chemical treatments of cardiolipin. Cardiolipin from bovine heart was purchased as ethanol solution from Sigma (Sigma product C 1649) and was stored at −20°C. To generate saturated molecular species, cardiolipin was treated with hydrogen in the presence of the catalyst PtO₂. Ten milligram of the catalyst were added to 0.2 mL of methanol/acetonitrile (1:1) and the suspension was bubbled with H₂ for 5 min. Then, 2.5 mg of cardiolipin were dissolved in 1 mL methanol/acetonitrile (1:1) and subsequently mixed with the H₂/PtO₂ suspension. Bubbling with H₂ was continued for 40 min on a heating block at 37°C. After hydrogenation, the catalyst was removed by centrifugation and filtration over glass wool. Hydrogenated cardiolipin was further purified by water/chloroform phase partitioning. Oxidized cardiolipin was generated by treatment with cytochrome c (15). To this end, 835 μg of cardiolipin were dried and resuspended in 2 mL of phosphate-buffered saline using a sonication water bath. Cytochrome c from horse heart was added to a final concentration of 10 μmol/L and the solution was incubated in a water bath at 37°C for 90 min. After the reaction, cardiolipin was recovered by chloroform/methanol extraction (16). Alternatively, cardiolipin was oxidized in aqueous solutions containing 1.5 mmol/L tert-butylhydroperoxide and CuSO₄ in concentrations ranging from 5 to 20 μmol/L. Cardiolipin was also oxidized by exposure to air. In this case, a thin film of cardiolipin was generated in a glass tube by slow evaporation of ethanol and the film was incubated at room temperature for 15 h.

High performance liquid chromatography (HPLC) of cardiolipin. Cardiolipin was resolved by reversed-phase chromatography to analyze the pattern of molecular species and to purify individual molecular species. The method was also used for quantification of cardiolipin. Up to 300 μg of cardiolipin were loaded on a Nucleosil C₁₈ (5 μm) column (length 250 mm, diameter 3.2 mm) using a 20 μL loop injector. A gradient was run from 10% solvent B to 90% solvent B within 40 min, using the solvent system described by Teng and Smith (17). Solvent A contained methanol, acetonitrile, and 10 mmol/L aqueous potassium phosphate (pH 7.46) at a volume ratio of 3:6:1. Solvent B contained methanol and acetonitrile at a ratio of 2:3. The flow rate was 1.0 mL/min. Chromatograms were recorded by measuring absorbance at 205 and 232 nm simultaneously, using the SPD-10AV dual wavelength UV-VIS detector from Shimadzu. Data were collected and processed by the Shimadzu CLASS VP software running on a desktop computer.

Electrospray ionization mass spectrometry. Electrospray ionization mass spectra were obtained on a Quattro II triple quadrupole mass spectrometer (Micromass, Beverly, MA) fitted with the standard electrospray probe. Samples were diluted to approximately 0.01 mmol/L in chloroform/methanol/water (11:8:2, v/v/v), containing 0.8 mmol/L ammonium bicarbonate. Solutions were infused via a syringe pump (Harvard Apparatus, Natick, MA) at a rate of 5 mL/min. The capillary voltage was set to 2.8 kV, the cone voltage typically to 35 V, and the source temperature to 65°C. In tandem mass spectrometric experiments, argon at a pressure of 3.6 × 10⁻⁵ mbar was used as collision gas with the collision energy set to 54 V.

Gas chromatographic analysis of fatty acids. Individual peaks of cardiolipin were separated by HPLC and collected for analysis of fatty acid patterns. After evaporation of the solvent, residues were resuspended by addition of 75 μL of toluene and 1 mL of methanol/HCl (0.5 mol/L). To transmethylate fatty acids, the mixture was incubated at 90°C over night in sealed derivatization tubes. The reaction was stopped by addition of 1 mL of NaHCO₃ solution (100 g/L) and fatty acid methyl esters were extracted into n-hexane. The extracts were dried under a stream of nitrogen and residues were dissolved in a few μL of tolulene. Fatty acid methyl esters were analyzed by gas chromatography on a SP-2330 capillary column (30 m × 0.32 mm × 0.2 μm) from Supelco. The carrier gas was helium and the column temperature was 185°C. A GC-17A Shimadzu instrument, equipped with a flame ionization detector, was used. The instrument was calibrated with commercial standards of fatty acid methyl esters. Data were collected and analyzed by the Shimadzu CLASS VP software.

Results

Behavior of cardiolipin on microtitre plates. Cardiolipin was applied to microtitre plates in ethanol and the solvent was allowed to evaporate. This was done in the same way as coating is routinely performed for the assay of antiphospholipid antibodies. After various periods of time at room temperature under normal atmosphere, the wells were

Fig. 1 Recovery of cardiolipin from microtitre plates. Four nanomoles of cardiolipin (in 10 μL ethanol) were added to each well of a microtitre plate and the plate was kept at ambient temperature, allowing the solvent to evaporate. After the indicated incubation intervals, wells were extracted with ethanol (0.1 mL/well). Extracts of 24 wells were pooled for measurement of cardiolipin (CL), organic phosphorus (P), and free fatty acids (FFA). Cardiolipin was measured by HPLC. Phosphorus was measured colorimetrically after digestion with HClO₄ (31), and divided by 2 to adjust for stochiometry. Free fatty acids were measured by quantitative gas chromatography with margaric acid as internal standard. Data are means with standard errors of mean of triplicate determinations.

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re-extracted with ethanol and the extracts were analyzed by HPLC to measure cardiolipin. Recovery of cardiolipin gradually declined during the first 5 h of incubation on the plate (Fig. 1). Failure to recover cardiolipin was caused by degradation rather than irreversible binding, because the phosphate groups remained fully extractable. The degradation product eluted near the solvent peak in reversed-phase HPLC and had a much higher absorbance ratio A232/A205 than parent cardiolipin (675 ± 47 10^{-5} versus 1.6 ± 0.4 10^{-5}, n = 3), indicating the generation of conjugated double bonds. The new compound was not lyso-cardiolipin because the amount of free fatty acids did not increase (Fig. 1).

Cardiolipin and its degradation product were further analyzed by electrospray ionization mass spectrometry. Native cardiolipin from bovine heart yielded three major signals, corresponding to the double charged anion (m/z 723.5), the single charged anion (m/z 1448), and the monosodium salt of the double charged anion (m/z 1470). The signals were centred around the m/z values expected for tetralinoleoyl-cardiolipin, the predominant molecular species (Fig. 2A). The degradation product, obtained by incubation of cardiolipin on microtitre plates, showed multiple derivative peaks with higher molecular masses in both the double charged and the single charged region (Fig. 2B). The derivative peaks of double charged ions were 8 m/z units apart, suggesting a series of progressively oxidized cardiolipins (Fig. 2C). The most abundant oxidation products were peroxo-tetralinoleoyl-cardiolipin (m/z 739.5), bis(peroxy)-tetralinoleoyl-cardiolipin (m/z 755.5), and tris(peroxy)-tetralinoleoyl-cardiolipin (m/z 771.5).

Confirmation of the structures was obtained from fragmentation patterns observed in collision induced decomposition by tandem mass spectrometry. In the collision induced decomposition spectrum of the m/z 1448 ion, product ion peaks at m/z 830 and 750 corresponded to the loss of diacylglycerol and diacylglycerophosphate, respectively, from tetralinoleoyl-cardiolipin. Ion peaks at m/z 695 (dilinoleoyl-glycerophosphate), m/z 433 (monolinoleoyl-glycerophosphate), and m/z 279 (linoleate) were also observed. The same set of ions were also seen in the collision induced decomposition spectrum of the m/z 1512 species of oxidized cardiolipin, showing that most of the structural elements were common in the two parent ions. All other ions were assignable to various combinations of losses of O_{2} and H_{2}O, consistent with the structure of bis(peroxy)-tetralinoleoyl-cardiolipin (data not shown).
Effect of oxidative treatment of cardiolipin. Five hours of incubation of cardiolipin on microtitre plates had little effect on the IgG-specific immune reaction of APS serum (Fig. 3). However, at the same time this incubation produced nearly complete oxidation of cardiolipin (Fig. 1). Only when the incubation was extended over night, did we observe a decrease in immune reactivity. The same result was obtained when pure tetrailnoeoyl-cardiolipin (L₄-CL) was used instead of commercial bovine heart cardiolipin. The comparison was made because commercial cardiolipin may contain pre-oxidized species, which may blunt the effect of further oxidation on the plate. This was unlikely in purified L₄-CL.

Next, we added oxidizing reagent, consisting of CuSO₄ and tert-butylhydroperoxide, to cardiolipin coated on microtitre plates. This treatment did not affect IgG binding from APS serum (Fig. 4) while resulting in Cu²⁺-dose dependent increase in oxidized cardiolipin, ranging from 1 to 10% (determined by HPLC). Finally, cardiolipin was pre-treated with oxidizing agents before coating on microtitre plates. Mild oxidative treatment with Cu²⁺/tert-butylhydroperoxide or exposure to air (proportion of oxidized cardiolipin: 2-8%) did not affect immune reactivity. More severe oxidation with cytochrome c (proportion of oxidized cardiolipin: 19%) caused a slight reduction of immune reactivity (Table 1).

Effect of different molecular species of cardiolipin. Cardiolipin was resolved by HPLC into molecular species defined by specific combinations of fatty acids. Cardiolipin from bovine heart yielded four peaks (Fig. 5, left panel). The number of peaks increased to seven due to oxid.
The oxidized peaks (oxCLa-c) had a shorter retention time than native cardiolipin and showed relatively high absorbance at 232 nm, indicating the presence of conjugated double bonds.

To test the reaction of individual cardiolipins in the assay, we coated aliquots of HPLC fractions side by side on microtitre plates and performed the IgG-specific enzyme-linked immunosorbent assay with APS serum. This method generated an “immunochromatogram” that largely mimicked the pattern of the uv chromatogram of native cardiolipin, i.e. each species supported immune reactivity (Fig. 6, upper panel). This was also true for oxidized species, generated by cytochrome c (Fig. 6, medium panel), as well as partly saturated species, generated by catalytic hydrogenation (Fig. 6, lower panel). In all we identified 12 cardiolipins that showed positive reaction in the immunoassay. These species were isolated and their fatty acid composition was analyzed by gas chromatography (Table 2). The species belonged to a homologous series of cardiolipins, each containing four C\textsubscript{18} residues and a total number of double bonds varying from 1 to 9. There was a linear relation between the number of double bonds and the logarithm of HPLC retention time (data not shown).

The IgG immune reaction of APS serum was determined with variable amounts of L\textsubscript{4}-CL, trilinoleoyl-oleoyl-cardiolipin (L\textsubscript{3}O-CL), oleoyl-tristearoyl-cardiolipin (O\textsubscript{3}S-CL), and oxCLb. L\textsubscript{4}-CL, L\textsubscript{3}O-CL, and oxCLb showed similar efficiency in the solid-phase assay, whereas Cardiolipin was treated with oxidants before it was coated on microtitre plates for immunoassay. Assays were performed in triplicates with the same APS serum sample. The amount of oxidized cardiolipin was measured by HPLC.

### Table 1
Effect of oxidative treatment of cardiolipin on immunoassay of APS serum

<table>
<thead>
<tr>
<th>Oxidative Treatment</th>
<th>Oxidized Cardiolipin</th>
<th>IgG Binding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(%)</td>
<td>(A\textsubscript{232} - A\textsubscript{205}) (%)</td>
</tr>
<tr>
<td>None</td>
<td>1</td>
<td>0.84±0.01</td>
</tr>
<tr>
<td>Exposure to air (1 hr)</td>
<td>2</td>
<td>0.85±0.03</td>
</tr>
<tr>
<td>Cu\textsuperscript{2+}(5 \textmu M)/tetr-BuOOH</td>
<td>4</td>
<td>0.81±0.08</td>
</tr>
<tr>
<td>Cu\textsuperscript{2+}(20 \textmu M)/tetr-BuOOH</td>
<td>8</td>
<td>0.76±0.08</td>
</tr>
<tr>
<td>Cytochrome c</td>
<td>19</td>
<td>0.55±0.01</td>
</tr>
</tbody>
</table>

- Cardiolipin was treated with oxidants before it was coated on microtitre plates for immunoassay. Assays were performed in triplicates with the same APS serum sample. The amount of oxidized cardiolipin was measured by HPLC.

### Table 2
Identification of molecular species by fatty acid analysis

<table>
<thead>
<tr>
<th>Peak</th>
<th>Fatty Acid Composition (mass %)</th>
<th>16:0</th>
<th>16:1</th>
<th>18:0</th>
<th>18:1</th>
<th>18:2</th>
<th>20:0</th>
<th>20:1</th>
<th>20:2</th>
<th>20:3</th>
<th>20:4</th>
<th>A\textsubscript{232} / A\textsubscript{205} (10\textsuperscript{3})</th>
<th>Molecular Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>oxCL a</td>
<td></td>
<td>5.0</td>
<td>tr.</td>
<td>6.5</td>
<td>19.9</td>
<td>62.1</td>
<td>6.5</td>
<td>n.d.</td>
<td>tr.</td>
<td>n.d.</td>
<td>103</td>
<td>n.k.</td>
<td></td>
</tr>
<tr>
<td>oxCL b</td>
<td></td>
<td>7.6</td>
<td>tr.</td>
<td>5.4</td>
<td>13.8</td>
<td>61.0</td>
<td>11.6</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.5</td>
<td>n.d.</td>
<td>310</td>
<td>n.k.</td>
</tr>
<tr>
<td>oxCL c</td>
<td></td>
<td>12.2</td>
<td>3.8</td>
<td>11.0</td>
<td>30.6</td>
<td>32.9</td>
<td>9.4</td>
<td>n.d.</td>
<td>n.d.</td>
<td>tr.</td>
<td>n.d.</td>
<td>230</td>
<td>n.k.</td>
</tr>
<tr>
<td>CL-1</td>
<td></td>
<td>3.8</td>
<td>tr.</td>
<td>3.8</td>
<td>3.7</td>
<td>67.3</td>
<td>21.0</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.4</td>
<td>53</td>
<td>EL\textsubscript{3}</td>
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<tr>
<td>CL-2</td>
<td></td>
<td>0.3</td>
<td>0.5</td>
<td>0.5</td>
<td>0.4</td>
<td>97.3</td>
<td>0.2</td>
<td>n.d.</td>
<td>tr.</td>
<td>n.d.</td>
<td>0.7</td>
<td>0.1</td>
<td>L\textsubscript{4}</td>
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<td>CL-3</td>
<td></td>
<td>6.2</td>
<td>0.1</td>
<td>0.9</td>
<td>16.9</td>
<td>73.7</td>
<td>0.2</td>
<td>n.d.</td>
<td>0.3</td>
<td>0.9</td>
<td>0.7</td>
<td>0.2</td>
<td>2</td>
</tr>
<tr>
<td>CL-4</td>
<td></td>
<td>3.7</td>
<td>n.d.</td>
<td>10.4</td>
<td>25.3</td>
<td>57.9</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.5</td>
<td>0.8</td>
<td>0.8</td>
<td>0.6</td>
<td>0</td>
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<tr>
<td>CL-5</td>
<td></td>
<td>6.0</td>
<td>n.d.</td>
<td>16.0</td>
<td>40.9</td>
<td>29.6</td>
<td>n.d.</td>
<td>tr.</td>
<td>2.3</td>
<td>1.2</td>
<td>2.4</td>
<td>1.6</td>
<td>0</td>
</tr>
<tr>
<td>CL-6</td>
<td></td>
<td>10.0</td>
<td>n.d.</td>
<td>14.5</td>
<td>62.1</td>
<td>2.9</td>
<td>n.d.</td>
<td>n.d.</td>
<td>10.5</td>
<td>n.d.</td>
<td>0.7</td>
<td>n.d.</td>
<td>0</td>
</tr>
<tr>
<td>CL-7</td>
<td></td>
<td>3.3</td>
<td>tr.</td>
<td>25.5</td>
<td>68.3</td>
<td>0.3</td>
<td>n.d.</td>
<td>tr.</td>
<td>2.2</td>
<td>0.5</td>
<td>tr.</td>
<td>0.2</td>
<td>0</td>
</tr>
<tr>
<td>CL-8</td>
<td></td>
<td>2.3</td>
<td>0.4</td>
<td>50.2</td>
<td>46.0</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>1.2</td>
<td>n.d.</td>
<td>tr.</td>
<td>0.2</td>
<td>0</td>
</tr>
<tr>
<td>CL-9</td>
<td></td>
<td>1.9</td>
<td>1.1</td>
<td>71.3</td>
<td>24.6</td>
<td>0.2</td>
<td>n.d.</td>
<td>tr.</td>
<td>0.7</td>
<td>0.2</td>
<td>n.d.</td>
<td>0.2</td>
<td>OS\textsubscript{3}</td>
</tr>
</tbody>
</table>

The peaks shown in Figs. 5–6 were analyzed for fatty acid composition. Data are means of duplicate determinations. Absorbance ratios $A_{232}/A_{205}$ were calculated from HPLC recordings. Fatty acids are noted as carbon number: double bond number. Abbreviations: E, linolenoyl (18:3); L, linoleoyl (18:2); O, oleoyl (18:1); S, stearoyl (18:0); n.d., not detectable; tr., trace amount; n.k., not known.
Cardiolipin is a unique dimeric phospholipid of mitochondrial membranes (18) and is widely used in the solid-phase assay of antiphospholipid antibodies. The abundance of polyunsaturated fatty acids in cardiolipin has led to the proposal that oxidative modifications of cardiolipin give rise to the formation of neo-epitopes (9). In reference to this idea, we measured the immune reaction with a number of distinct molecular species each characterized by a specific combination of fatty acids. We found that all cardiolipins, including oxidized, unsaturated, and saturated species, were capable to elicit an immune reaction with APS serum (Fig. 6). In fact the elution pattern, monitored by immune reaction, looked very similar to the one monitored by absorbance (compare Figs. 5 and 6).

Solid-phase assays for antiphospholipid antibodies are a mainstay in the diagnosis and management of APS and lupus erythematosus (19). Unfortunately this test is susceptible to a number of factors that are difficult to control, i.e. interlaboratory variation is very high (20-22). Coating of cardiolipin on microtitre plates may introduce variation because of the potential for lipid degradation with high dependency on ambient conditions. Indeed, products of lipid oxidation were found to accumulate on microtitre plates during coating (9, 23). Thus, the question arises as to whether lipid oxidation affects results obtained with the solid-phase assay. We found that, within 5 h of coating, cardiolipin was completely converted into a series of hydroperoxy analogues. Hydroperoxy-L_{4-CL}, bis(hydroperoxy)L_{4-CL}, and tris(hydroperoxy)L_{4-CL} were the most abundant products (Figs. 1-2). However we did not detect any significant change in immune reactivity during this period of time (Fig. 3). This corresponded to the observation that oxidized and non-oxidized species of cardiolipin had the same efficiency in the assay (Fig. 7). Furthermore, oxidative treatment of cardiolipin, either before or after coating, did not effectively alter antibody binding (Fig. 4, Table 1). Only over-night incubation on microtitre plates (Fig. 3) or very strong oxidative treatment (Table 1) did result in significant loss of immune reactivity. This effect is most likely caused by further decomposition of oxidized cardiolipin. For instance, dilyso-cardiolipin was formed as a result of extensive oxidation (data not shown). Dilyso-cardiolipin does not support the immune reaction (24).

Hörkkö et al. suggested that the anticardiolipin assay measures antibodies directed against oxidized epitopes (9, 10). This is in line with related evidence demonstrating the presence of autoantibodies against oxidized lipoproteins (25), and oxidized phosphatidylcholine (26). However, in contrast to Hörkkö et al. (9, 10) we did not find that cardiolipin oxidation increased immune reactivity or was even a precondition for antibody binding. To explain this discrepancy, the following points must be considered. First, the source of cardiolipin and the kind of microtitre plate may affect the immune reaction. In particular, the pre-existing degree of oxidation in the cardiolipin preparation may be important. However, the fact that commercial bovine heart cardiolipin behaved exactly like freshly purified L_{4-CL} was reproduced with eight different patient sera (Fig. 7, right panel). One crucial difference between L_{4-CL} and OS_{3-CL} was susceptibility to oxidation, as demonstrated by HPLC analysis of extracts from microtitre plates. Whereas L_{4-CL} was largely oxidized after 5 h of incubation on microtitre plates (recovery = 9 ± 4%, n = 3), OS_{3-CL} was resistant to oxidation (recovery = 95 ± 5%, n = 3).

Immune reactions of three cardiolipin species were also measured with the antibody A1.72, a monoclonal IgM derived from lupus-prone mice (14). Like human IgG, A1.72 showed maximal reactivity with L_{4-CL} and L_{0-CL}, and somewhat lower reactivity with OS_{3-CL}. Oxidation of cardiolipin, either by air or by Cu^{2+}/tert-butylhydroperoxide, did not alter A1.72 binding (Fig. 8).

Discussion

Cardiolipin is a unique dimeric phospholipid of mitochondrial membranes (18) and is widely used in the solid-phase assay of antiphospholipid antibodies. The abundance of polyunsaturated fatty acids in cardiolipin has led to the proposal that oxidative modifications of cardiolipin give rise to the formation of neo-epitopes (9). In reference to this idea, we measured the immune reaction with a number of distinct molecular species each characterized by a specific combination of fatty acids. We found that all cardiolipins, including oxidized, unsaturated, and saturated species, were capable to elicit an immune reaction with APS serum (Fig. 6). In fact the elution pattern, monitored by immune reaction, looked very similar to the one monitored by absorbance (compare Figs. 5 and 6).

Solid-phase assays for antiphospholipid antibodies are a mainstay in the diagnosis and management of APS and lupus erythematosus (19). Unfortunately this test is susceptible to a number of factors that are difficult to control, i.e. interlaboratory variation is very high (20-22). Coating of cardiolipin on microtitre plates may introduce variation because of the potential for lipid degradation with high dependency on ambient conditions. Indeed, products of lipid oxidation were found to accumulate on microtitre plates during coating (9, 23). Thus, the question arises as to whether lipid oxidation affects results obtained with the solid-phase assay. We found that, within 5 h of coating, cardiolipin was completely converted into a series of hydroperoxy analogues. Hydroperoxy-L_{4-CL}, bis(hydroperoxy)L_{4-CL}, and tris(hydroperoxy)L_{4-CL} were the most abundant products (Figs. 1-2). However we did not detect any significant change in immune reactivity during this period of time (Fig. 3). This corresponded to the observation that oxidized and non-oxidized species of cardiolipin had the same efficiency in the assay (Fig. 7). Furthermore, oxidative treatment of cardiolipin, either before or after coating, did not effectively alter antibody binding (Fig. 4, Table 1). Only over-night incubation on microtitre plates (Fig. 3) or very strong oxidative treatment (Table 1) did result in significant loss of immune reactivity. This effect is most likely caused by further decomposition of oxidized cardiolipin. For instance, dilyso-cardiolipin was formed as a result of extensive oxidation (data not shown). Dilyso-cardiolipin does not support the immune reaction (24).

Hörkkö et al. suggested that the anticardiolipin assay measures antibodies directed against oxidized epitopes (9, 10). This is in line with related evidence demonstrating the presence of autoantibodies against oxidized lipoproteins (25), and oxidized phosphatidylcholine (26). However, in contrast to Hörkkö et al. (9, 10) we did not find that cardiolipin oxidation increased immune reactivity or was even a precondition for antibody binding. To explain this discrepancy, the following points must be considered. First, the source of cardiolipin and the kind of microtitre plate may affect the immune reaction. In particular, the pre-existing degree of oxidation in the cardiolipin preparation may be important. However, the fact that commercial bovine heart cardiolipin behaved exactly like freshly purified L_{4-CL} was reproduced with eight different patient sera (Fig. 7, right panel). One crucial difference between L_{4-CL} and OS_{3-CL} was susceptibility to oxidation, as demonstrated by HPLC analysis of extracts from microtitre plates. Whereas L_{4-CL} was largely oxidized after 5 h of incubation on microtitre plates (recovery = 9 ± 4%, n = 3), OS_{3-CL} was resistant to oxidation (recovery = 95 ± 5%, n = 3).

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OS_{3-CL} was less effective (Fig. 7, left panel). The pattern L_{4-CL} = L_{0-CL} = oxCLb > OS_{3-CL} was reproduced with eight different patient sera (Fig. 7, right panel). One crucial difference between L_{4-CL} and OS_{3-CL} was susceptibility to oxidation, as demonstrated by HPLC analysis of extracts from microtitre plates. Whereas L_{4-CL} was largely oxidized after 5 h of incubation on microtitre plates (recovery = 9 ± 4%, n = 3), OS_{3-CL} was resistant to oxidation (recovery = 95 ± 5%, n = 3).

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resistant to oxidation (9). However, there is evidence showing that the immune reaction is supported by tetramyristoyl-cardiolipin, which is also saturated and therefore resistant to oxidation (23, 27). In this paper we found significant antibody binding in the presence of OS3-CL (Fig. 7). OS3-CL is resistant to oxidation because it contains only a single double bond, whereas lipid oxidation can only proceed in the presence of two or more double bonds (28). We confirmed this by demonstrating that OS3-CL was not chemically altered on microtitre plates before the immunoassay either by Cu2+ (20 μmol/L)/tert-butylhydroperoxide (1.5 mmol/L) or by exposure to air for 5 h. Two micrograms of cardiolipin were applied per well. Data are means with standard errors of mean of quadruplicate determinations.

Fig. 7 Immune reactivity of different molecular species of cardiolipin. Left panel: Dependence of the immune reaction on the amount of cardiolipin per well. Serum from one APS patient was used. Right panel: Immune reaction of eight different APS sera in the presence of 2 μg cardiolipin per well.

In summary, we found that incubation of CL on microtitre plates, for the purpose of solid-phase immunoassays, may indeed produce various hydroperoxy derivatives of the parent lipid. However, oxidation of CL did not alter the immune reaction of both APS serum and monoclonal IgM from lupus-prone mice. Instead the solid-phase assay displayed little specificity for molecular acyl species of cardiolipin.

Fig. 8 Immune reactivity of monoclonal IgM (A1.72) from lupus-prone mice with different cardiolipins. Binding of A1.72 was measured with tetralinoleoyl-cardiolipin (L4-CL), trilinoleoyl-oleoyl-cardiolipin (L3O-CL), oleyl-tristearoyl-cardiolipin (OS3-CL), and bovine heart cardiolipin. Bovine heart cardiolipin was treated on microtitre plates before the immunoassay either by Cu2+ (20 μmol/L)/tert-butylhydroperoxide (1.5 mmol/L) or by exposure to air for 5 h. Two micrograms of cardiolipin were applied per well. Data are means with standard errors of mean of quadruplicate determinations.

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